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Yuanmin Wang

H. Peter Lu

Bowling Green State University, hplu@bgsu.edu

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Bunching Effect in Single-Molecule T4 Lysozyme Nonequilibrium Conformational Dynamics under Enzymatic Reactions

Yuanmin Wang and H. Peter Lu*

Bowling Green State University, Center for Photochemical Sciences, Department of Chemistry, Bowling Green, Ohio 43403

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The bunching effect, implying that conformational motion times tend to bunch in a finite and narrow time window, is observed and identified to be associated with substrate–enzyme complex formation in T4 lysozyme conformational dynamics under enzymatic reactions. Using single-molecule fluorescence spectroscopy, we have probed T4 lysozyme conformational motions under the hydrolysis reaction of polysaccharide of *E. coli* B cell walls by monitoring the fluorescence resonant energy transfer (FRET) between a donor–acceptor probe pair tethered to T4 lysozyme domains involving open–close hinge-bending motions. On the basis of the single-molecule spectroscopic results, molecular dynamics simulation, and a random walk model analysis, multiple intermediate states have been estimated in the evolution of T4 lysozyme enzymatic reaction active complex formation (Chen, Y.; Hu, D.; Vorpagel, E. R.; Lu, H. P. Probing single-molecule T4 lysozyme conformational dynamics by intramolecular fluorescence energy transfer. *J. Phys. Chem. B* **2003**, *107*, 7947–7956). In this Article, we report progress on the analysis of the reported experimental results, and we have identified the bunching effect of the substrate–enzyme active complex formation time in T4 lysozyme enzymatic reactions. We show that the bunching effect, a dynamic behavior observed for the catalytic hinge-bending conformational motions of T4 lysozyme, is a convoluted outcome of multiple consecutive Poisson rate processes that are defined by protein functional motions under substrate–enzyme interactions; i.e., convoluted multiple Poisson rate processes give rise to the bunching effect in the enzymatic reaction dynamics. We suggest that the bunching effect is likely common in protein conformational dynamics involved in conformation-gated protein functions.

Introduction

Conformational motions are essential for the catalytic functions of enzymes.^{1–18} Involved in functionally conformational motions, an enzyme adjusts its physical and chemical flexibility toward an active state (ES*), which consists of the binding complex of an enzyme (E) and a substrate (S) for a specific catalytic reaction. It is reported that the enzyme may experience a set of intermediate states or transient states before reaching a reactive enzyme–substrate complex state.^{3,6,8,11,16,19–25} The conformational transition has been reported to occur at a time scale of microseconds to milliseconds, which is consistent with the reality that many functionally biological processes happen in this time regime.^{16,26–29} The enzyme–substrate intermediate states have been indentified by various ensemble-averaged measurements, such as X-ray crystallography,²⁵ NMR,³⁰ and ultrafast pump–probe absorption spectroscopy.²⁴ Because conformational motions reflect the real-time picture of the enzyme evolving from inactive states to active states of the catalytic reaction, to get the molecular level insights of the transition dynamics is critical for a further understanding of enzymatic reaction dynamics. Single-molecule spectroscopy has been an effective approach for probing fluctuation statistics, conformational gating,^{31,32} and function conformational dynamics of enzymes.^{1,11–13,16–18,21,23,33–36} In recent years, both experimental and theoretical single-molecule works have shed light on multiple intermediate states of single-molecule enzymatic enzyme–substrate formation dynamics^{16,37} and energy landscapes.^{11,12,23}

In complex rate processes, such as a nonequilibrium protein reaction process, with the local environmental fluctuation, nonexponential dynamics can be observed in reaction dynamics and conformational dynamics.^{38,39} For the multiple interconverting conformational states related to the fluctuating catalytic reactivity,⁴⁰ a specific function has been applied to interpret the continuous conformational states.³⁹ For a biochemical process, multiple rate-limiting steps lead to nonexponential turnover time distributions.⁴¹ On the basis of the study of single-molecule fluorogenic product generation enzymatic dynamics, it has been demonstrated that power-law temporal behavior of the activity fluctuation statistics can be observed because of the slow barrier-height fluctuations in single-enzyme reactions.⁴² Although the conformational dynamics has been investigated by statistical approaches in the single-molecule enzymatic reaction, it is still difficult to resolve the sequential multiple conformational dynamics. As hidden events, specific properties of the enzyme conformational motions are expected to be probed by modeling and simulation combined with the single-molecule experiment such as fluorescence resonant energy transfer (FRET) measurements.

Previously, we analyzed single-molecule T4 lysozyme conformational dynamics by FRET measurements probing the hinge-bending motions of the enzyme under enzymatic reaction conditions (Figure 1).^{16,37} We found that the overall enzymatic reaction rate constants are inhomogeneous from molecule to molecule and that multiple intermediate conformational states in the enzymatic reaction exist, as revealed by molecular dynamics simulation and the random-walk model based on our experimental results (Figure 2A,B).¹⁶ Here, presenting a progress

* To whom correspondence should be addressed. E-mail: hplu@bgsu.edu.

on our single-molecule data analysis and interpretation, we report a new insight into the single-molecule conformational dynamics of T4 lysozyme: a bunching effect associated with forming an active conformation state, ES*. The bunching effect suggests that the enzymatic conformational motion times in forming the ES* state tend to be distributed in a finite and narrow time window measured by our single-molecule spectroscopy experiments.

Model Analysis and Approaches

Simulation of the Single-Molecule T4 Lysozyme Nonequilibrium Conformational Dynamics under Enzymatic Reactions. In our study, the enzyme–substrate active-state formation time, t_{ES^*} , is defined as the time interval during which the active site opens due to substrate insertion and decreases as the active site closes to form an active enzyme–substrate complex (Figure 2B). In the single-molecule FRET measurements of T4 lysozyme enzymatic reaction, t_{ES^*} can be deduced from the slow wiggling event of the fluorescence trajectory of the donor (Figure 2B). To simulate the t_{ES^*} probability distributions for the T4 lysozyme enzymatic reaction, a modified Michaelis–Menton mechanism of the enzymatic reaction (Figure 2C) was first proposed on the basis of the experimental data and MD simulation. The probability function $P(t_n)$ (for six intermediate steps, $n = 1, 2, 3, 4, 5, 6$) of the formation times was then obtained from the convolution model and calculation. On the basis of the probability functions, random numbers were generated for each function. Then, random numbers were made to be unordered and form the simulated formation-time trajectories. A specific number of the simulated formation times was used to build up the histograms and two-dimensional joint probability distributions, i.e., $f(t_i, t_{i+j})$, which is defined by

$$f(t_i, t_{i+j}) = P((k-1)\Delta x \leq t_i < k\Delta x, (k'-1)\Delta y \leq t_{i+j} < k'\Delta y) \\ j = 1, 2, 3, \dots, N \quad (1)$$

where P is a probability function, j is the separation time index between two formation times, $x \in \{0, \Delta x, \dots, k\Delta x, \dots, 50\}$ and $y \in \{0, \Delta y, \dots, k'\Delta y, \dots, 50\}$ as the x and y axis of the 2D joint probability distributions (unit: ms; $\Delta x = \Delta y$; $k = 1, 2, 3, \dots, N$; $k' = 1, 2, 3, \dots, N$), and t_i and t_{i+j} are a pair of formation times separated by index j . For any pairs of formation times (t_i, t_{i+j}) , if they meet $(k-1)\Delta x \leq t_i < k\Delta x$ and $(k'-1)\Delta y \leq t_{i+j} < k'\Delta y$ in x and y dimension, respectively, the amount of these pairs (in the specific area of Δx by Δy) will be counted as probability in the z dimension and shown by a color bar in the 2D joint probability distributions.

Single-Molecule FRET Spectroscopy Probing T4 Lysozyme Catalytic Conformational Dynamics. A detailed description of site-specific donor–acceptor dye labeling of T4 lysozyme and the corresponding single-molecule FRET measurements can be found in one of our earlier publications.¹⁶ Briefly, site-specific donor–acceptor dye labeling was conducted for the FRET measurements. FRET labeled T4 lysozyme was covalently linked to a silanized and hydrocarbon molecule coated (1:10000) glass coverslip by the bifunctional linker, SIAXX (Molecular Probes, Inc.). A confocal microscope with 532 nm laser excitation from a diode-pumped solid-state CW (continuous wave) laser was used to image the individual T4 lysozyme molecules and measure the single-molecule fluorescence intensity trajectories. The donor TMR and acceptor Texas Red emissions were detected separately by a pair of avalanche photodiode detectors after passing through a 570 nm band-pass

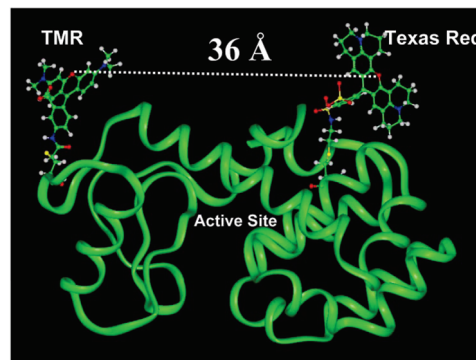


Figure 1. Crystal structure of the wild-type T4 lysozyme (3LZM, from the Protein Data Bank). A pair of tetramethylrhodamine and Texas Red dye molecules is covalently tethered to the cysteine 54 and cysteine 97 residues of a T4 lysozyme protein molecule as donor and acceptor for single-molecule FRET measurements. Single-molecule FRET is sensitive to the distance changes between the two domains involved in hinge-bending motions.

filter (bandwidth: 20 nm) and a 615 nm long-pass filter, respectively.^{16,37}

Results and Discussion

Figure 1 shows the crystal structure of wild-type T4 lysozyme with a pair of FRET fluorophores (tetramethylrhodamine and Texas Red) capable of probing the open–close hinge-bending motions of two domains of the T4 lysozyme enzyme molecules. The substrate insertion into the active site and formation of the active enzyme–substrate complex, associated with the active site conformation open–close motions, are probed by the anticorrelated fluorescence intensity changes of the donor and acceptor dyes. The single-molecule conformational fluctuations measured without or with the substrate (polysaccharide of *E. coli* cell walls) show different fluctuation rates. The fluctuation rates calculated from the single-molecule conformational change time trajectories for the former is dominated by faster fluctuations with a broadly distributed rate background; in contrast, the conformational fluctuation rates for the latter are statistically slower and narrowly distributed due to the substrate binding induced hinge-bending motions of the enzyme in forming the reactive complex states, ES*. It is likely that the substrate binding selectively shifted the equilibrium among the fluctuating conformations of the enzyme which energetically and dynamically regulated the hinge-bending motions into a specific conformational fluctuation time range. The specific conformational motions involve multiple intermediate states, which can be essentially described by a conformation selection mechanism.^{43–47} Our previous model analysis of the experimental data indicated that there are at least 5–6 intermediate conformation states involved in ES* formation, $E + S \rightarrow ES_1 \rightarrow \dots \rightarrow ES^*$. Here, ES_1 represents a nonspecifically bound complex state and ES^* represents a specifically bound complex ready to react.

Considering that there are multiple intermediate conformational states formed in the T4 lysozyme conformational motion dynamics,^{16,37} and based on the schematic presentation of an enzymatic reaction (Figure 2A) as well as on the hinge-bending motion characteristics (Figure 2B), we suggest a modified Michaelis–Menton mechanism for the T4 lysozyme enzymatic reaction (eq 2).



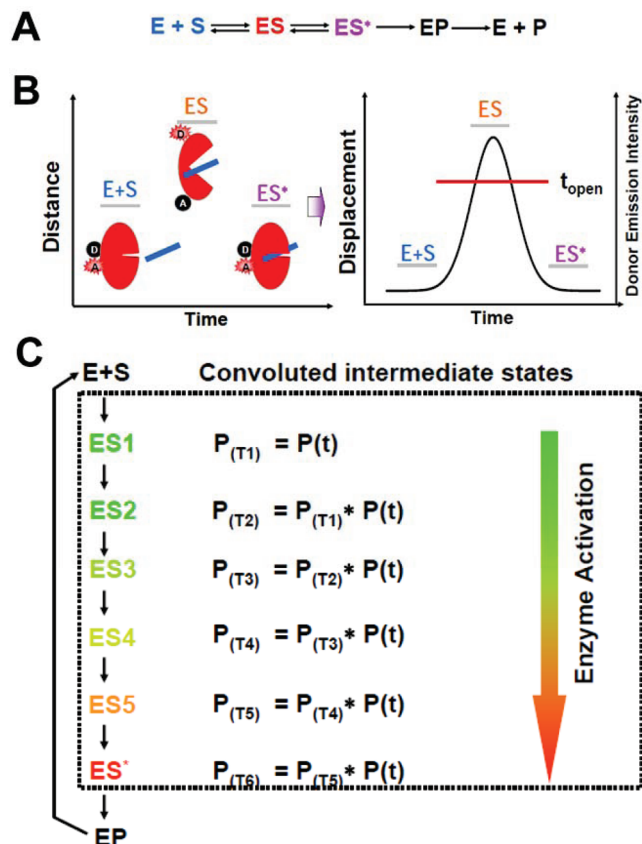


Figure 2. Modified Michaelis–Menton mechanism for T4 lysozyme conformational dynamics. (A) Schematic presentation of the enzymatic reaction. E, S, and P represent the enzyme, substrate, and product, respectively. (B) Correlation between the enzyme hinge-bending motion and the variation of donor fluorescence intensity in one ES*-formation time. (C) Scheme of the enzyme reaction and the formation-time probabilities of multiple convoluted intermediate states. From E+S to ES*, the enzyme experiences multiple intermediate states and finally reaches the active state, ES*. $P(t)$ is the probability distribution of the step times. $P_{(Tn)}$ ($n = 1, 2, 3, 4, 5, 6$) are the formation-time probabilities for the intermediate states, and ES* is readied for the enzymatic reaction.

where $n = 1, 2, 3, \dots, N$. The enzymatic reaction is primarily driven by the electrostatic attraction between the positively charged surface of T4 lysozyme's surface amino acid residues (arginine and lysine) and the negatively charged polysaccharide substrate. Being propelled by the electrostatic attraction, the positively charged front surface of the T4 lysozyme and the negatively charged substrate approach each other to form enzyme–substrate nonspecifically bounded complex states, ES_{*n*}. During the enzyme–substrate interaction, both electrostatic attraction and hydrogen bonding^{16,22} act as main driving forces to form the final active complex, ES*. Considering the perturbation of the local environment and thermal effect, the step between two adjacent states (for example, from state ES_{*n-1*} to state ES_{*n*} or from the last intermediate state to the active state ES*) is a stochastic process, and the step time obeys Poisson statistics, showing a single exponential distribution as

$$P(t) = A[\exp(-t/\tau)] \quad (3)$$

where $P(t)$ is the probability distribution of the step times, τ is the averaged step time, and A is the distribution weight constant. The step time is the duration between two adjacent states, and it is different from the formation time of the intermediate states

or active state. In our model, the formation time is the convolution of step times. On the basis of our experimental data, the average formation time of the T4 lysozyme enzymatic reaction in forming the active complex ES* through eq 2 is 19.5 ms, and the enzymatic reaction experienced about six intermediate steps during the formation time.¹⁶ Since the conformational motions probed are primarily along the hinge-bending motion coordinate and driven by similar driving forces between enzyme and substrate, it is reasonable to assume that the substeps in forming the ES* are Poisson processes with similar rates, and the time distribution for each substep obeys eq 3. Therefore, it takes 3.25 ms averagely for a transition between two adjacent intermediate states in eq 2. On the other hand, the multiple and consecutive intermediate state transitions to form ES* are convoluted (Figure 2). Taking $\tau = 3.25$ ms, we deduce that the probability to form the first intermediate state (S_1) can be expressed as a Poisson rate process of $P_{(T1)} = P(t) = A[\exp(-t/3.25)]$. For the convoluted intermediate state transition, one can get the probability function $P_{(Tn)}$ from the integral algorithm of eq 4. To calculate the convolution of function $f(t)$ and $g(t)$, the particular integral transform is

$$(f * g)(t) = \int_0^t f(v) \cdot g(t - v) dv \quad (4)$$

If two consecutive intermediate steps are involved in the hinge-bending motion, the probability function of the formation times is expressed as $P_{(T2)} = P_{(T1)} * P(t) = P(t) * P(t)$, based on eq 4, $P_{(T2)} = A^2\{t[\exp(-t/3.25)]\}$. For the T4 lysozyme enzymatic reaction, six intermediate steps are deduced from our experimental data based on a random walk model analysis.¹⁶ We then have $P_{(T6)} = A^6\{t^5[\exp(-t/3.25)]\}/120$. The general probability function is deduced to be

$$P_{(Tn)} = A^n \frac{t^{n-1}[\exp(-t/\tau)]}{(n-1)!} \quad (5)$$

where n ($1, 2, 3, \dots, N$) is the index of the intermediate steps; τ is the mean formation time of an intermediate state through a single-step rate process. The probability functions of the convoluted multiple intermediate states (or transient states) presented here can also be applied to other single-molecule enzyme conformational dynamics studies.

On the basis of the function $P_{(Tn)}$ ($n = 1, 2, 3, 4, 5, 6$) of eq 5, the distribution of conformational motion time for multiple consecutive intermediate steps in forming the active complex of ES* can be simulated. In Figure 3, A1, B1, C1, and D1 are the probability distributions of formation times for the enzyme involving one, two, four, and six intermediate steps, respectively; A2, B2, C2, and D2 are the corresponding two-dimension joint probability distributions of the adjacent formation times described as $f(t_i, t_{i+1})$. $f(t_i, t_{i+1})$ is constructed by adjacent conformational motion times of T4 lysozyme, forming a complex with the substrate in the enzymatic reaction. The histogram of the simulated formation times for only one intermediate step shows a typical Poisson distribution (Figure 3A1) and a characteristic wing feature in the 2D joint probability distribution (Figure 3A2), implying that there is no correlation and bunching effect in the formation times. This is consistent with the stochastic nature of the Poisson rate process. However, the histograms of simulated conformational motion times involved in multiple intermediate steps show a non-Poisson distribution and even Gaussian-like distributions (Figure

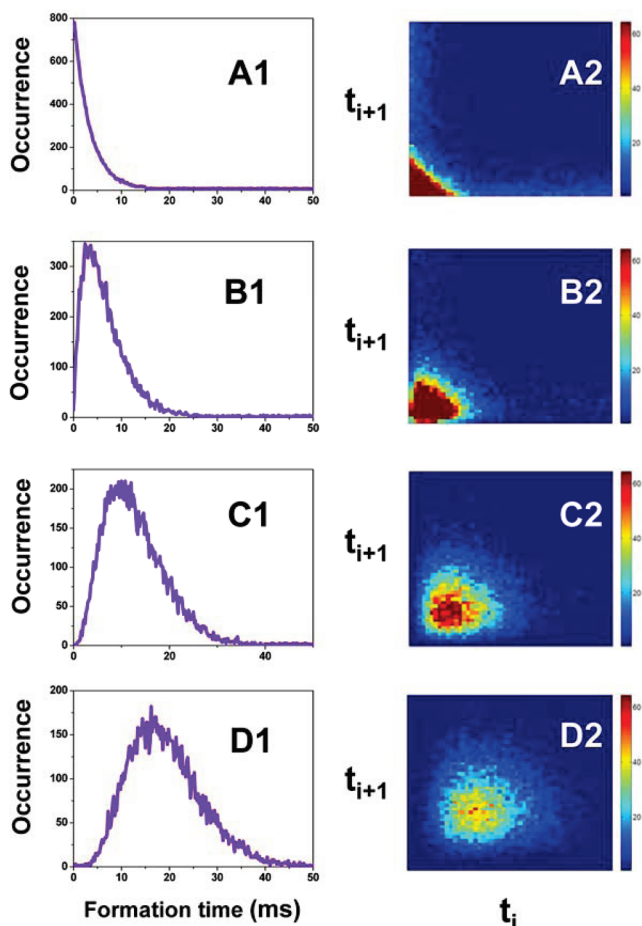


Figure 3. Simulated distribution of formation times (A1–D1) and corresponding two-dimensional joint probability distributions (A2–D2) of adjacent formation times for different intermediate steps. As a single step, A1 shows exponential distribution and A2 only shows a wing structure, implying one step is a Poisson process and there is no bunching effect. However, for the multiple steps such as 2, 4, and 6 steps, nonexponential distributions of the probabilities (in B1, C1, and D1) and the bunching structures (in B2, C2, and D2) are increasingly clear, implying the bunching nature in the open–close conformational motion times.

3B1–3D1). The simulated conformational motion times involving multiple intermediate states unambiguously show a bunching effect as evident by the aggregated data points at a narrow region along the diagonal direction in the 2D joint probability distributions (Figure 3B2–3D2). The observed bunching effect implies that the conformational motion time tends to be distributed in a finite and narrow time window as a Gaussian-like distribution with defined and comparable first and second moments. The existence of the bunching effect in the conformational motion time strongly suggests that the hinge-bending open–close conformational motions in enzymatic reaction turnover cycles optimize the physical and chemical flexibility of the enzyme to similar domain configurations for forming the same ES* complex in the catalytic hydrolysis reaction. The physical picture suggests strongly that the conformational dynamics of T4 lysozyme shows a characteristic behavior of enzyme conformation selection dynamics driven by substrate–enzyme interactions. This attribution is also consistent with our experimental evidence:¹⁶ (1) the fluctuation rates of the hinge-bending motions of single-molecule T4 lysozyme are dominated with fast fluctuations (faster than ms) under a broad rate background when there is no substrate, whereas the fluctuation rates show a Gaussian-like distribution at the ms time scale when there is

substrate interacting with the enzyme. (2) Under the same enzymatic reaction condition, the first and second moments of the $E + S \rightarrow ES \rightarrow \rightarrow ES^*$ formation time distributions are essentially homogeneous among the single T4 lysozyme molecules.

In a Poisson rate process, there should be no bunching among the stochastic conformational fluctuation times. However, a nonequilibrium rate process through a sequence of consecutive Poisson processes with comparable rates eventually produces a bunching effect within the overall time lapse for the overall multiple-step rate process. For T4 lysozyme's conformational dynamics, the physical nature of the bunching effect is associated with the functionally conformational motion mechanism⁶ involved in nonequilibrium conformational fluctuations.¹⁶ The characteristics of the nonequilibrium conformational fluctuation dynamics are experimentally observed by oscillatory fluctuations of the conformational open–close motions and the Gaussian-like formation time distributions.¹⁶ The conformational dynamics is significantly regulated by the interactions between the T4 lysozyme and the substrate in terms of electrostatic attraction and hydrogen bonding interactions, being associated with the formation of the active complex state of ES* (Figures 2 and 3) for the T4 lysozyme enzymatic reactions. For the formation of the first intermediate state, the distribution of the conformational motion time is stochastic and there is no bunching among the conformational motion times. However, with subsequent intermediate states being formed in consecutive multiple steps, the bunching effect appears for the formation times and in turn becomes more and more prominent. This is evident in the 2D joint probability distribution plot (Figure 3) as the conformational motion times are defined in a Gaussian-like distribution and gives a finite second moment of the distribution; accordingly, the conformational motion times show a significantly high probability in distribution around a specific time scale of ms along the diagonal direction in the 2D joint probability plots.

Figure 4A shows a distribution of experimental ES* formation times, t_{ES^*} , measured in our previously reported single-molecule T4 lysozyme enzymatic reaction experiments, in which six intermediate steps in forming the ES* active complex state are suggested. The average formation time associated with T4 lysozyme hinge-bending open–close motion in each enzymatic reaction turnover cycle, $\langle t_{ES^*} \rangle$, is measured as 19.5 ± 2 ms. Figure 4B shows our simulated data for six intermediate steps using eqs 2 and 5, in which $\langle t_{ES^*} \rangle$ is calculated to be 19.1 ± 2 ms. Unambiguously, the simulated data shows essentially the same ES* formation time distribution with a Gaussian-like profile and the mean value of t_{ES^*} (Figure 4B) compared with the experimental t_{ES^*} distribution (Figure 4A).

The bunching effect observed in T4 lysozyme enzymatic conformational dynamics is novel, and it is distinctively different from the previously reported memory effect in enzymatic reaction dynamics. The memory effect, an enzymatic reaction turnover time is dependent on its previous turnover time, has been extensively reported in recent years.^{1,6,7,19,36,39} The typical characteristics of a memory effect in dynamics is that a long turnover time is likely to be followed by a long one and a short turnover time is likely to be followed by a short one in enzymatic reaction. The quantitative description of the memory effect is to use an autocorrelation function of a single-molecule turnover time trajectory and the diagonal features in a two-dimensional joint probability distribution. On the basis of a number of recently published experimental results and simulations, slow functional conformational motion and equilibrium conformational fluctuations in enzymatic turnovers have been

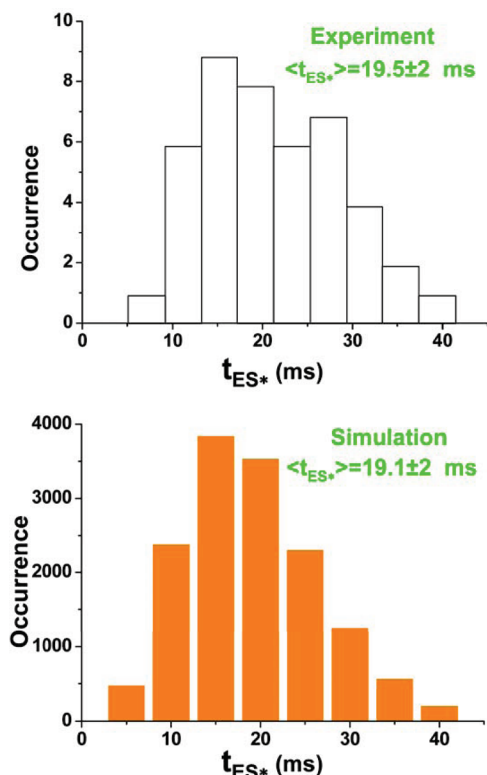


Figure 4. Histograms of the experimental¹⁶ and simulated formation times (t_{ES^*}) of the conformational motions in forming ES^* . The experimental data is deduced from a single T4 lysozyme fluorescence trajectory.¹⁶ t_{ES^*} is the duration time of each wiggle of the intensity trajectory above a threshold. The threshold is determined by 50% of the bimodal intensity distribution.¹⁶ The mean formation times, $\langle t_{ES^*} \rangle$, are 19.5 ± 2 and 19.1 ± 2 ms for the experiment and simulation, and the corresponding standard deviation of the formation time is 8.3 ± 2 and 7.5 ± 2 ms, respectively.

suggested to be responsible for memory effects in the enzymatic turnover cycles, such as for cholesterol oxidase.^{1,6,9} The bunching effect for enzymatic conformational dynamics reported here is related but significantly different from the previously reported memory effect. For a typical memory effect in enzyme conformational motions under an enzymatic reaction, the conformational motion times may cover a broad time scale of many folds along the diagonal direction in a two-dimensional joint probability distribution,⁷ and the second moment of the time distribution is not necessarily finite, as the time distribution is non-Gaussian and often Lévy or Lorenzin.^{48–50} However, in the bunching effect associated conformational dynamics presented in this study, the conformational motion times distribute in a relatively narrow range and the data points show bunching at a specific area along the diagonal direction in a two-dimensional joint probability distribution plot (Figures 3D1 and D2 and 4). The second moment of the time distribution is finite, and the time distribution is Gaussian or Gaussian-like. Furthermore, for the dynamics showing a memory effect, the 2D joint probability distribution, $f(t_i, t_{i+j})$, changes with the time separation index j : as j increases, the diagonal feature intensity decreases as the memory only exists for a finite time; however, for the bunching effect, the 2D joint probability distribution is essentially stationary with changing j , and the bunching effect demonstrates a stable impact on the enzyme function and dynamics as long as the external environment and reaction conditions are kept the same. The bunching effect often shows a strong regulation and controlling impact in conformation-gated protein function and dynamics. We suggest that the bunching effect and memory

effect, both as typical dynamic behavior of nonequilibrium enzymatic reaction dynamics, are two general behaviors that commonly occur in the enzymatic conformational dynamics, especially for conformational regulated enzymatic reactions. Their occurrence possibilities are determined by the nature of the conformational motions, which is regulated by the interaction between enzyme and substrate, such as geometrical constraints, solvent perturbation, electrostatic interaction, hydrophobicity, and binding modes.

The open–close hinge-bending conformational motions of T4 lysozyme in the formation of the ES^* active complex state involve multiple intermediate states (Figure 2). This nonequilibrium conformational fluctuation process is critical for T4 lysozyme to form the active substrate–enzyme complex state for the enzymatic reaction, and it is this process that defines the energy landscape for the enzyme active site coordinates and the immediately followed enzymatic reactions. The measured t_{ES^*} can also be characterized as the first-passage time for the formation of ES^* , assuming the conformational motions along paths of the coordinate space defined by the hinge-bending motion coordinates of the T4 lysozyme. In our model analysis, we chose the simplest assumption that the chain multiple steps involved in the hinge-bending conformational motions of T4 lysozyme are Poisson processes at the same time constant of 3.25 ms. In a more complex model assumption of that, the chain multiple Poisson rate steps have different time constants for individual steps; i.e., there is a distribution of time constants. In this more complex model, if an intermediate step in the chain of multiple-step Poisson rate process is significantly faster than other processes, then the contribution of the faster process is negligible for overall first-passage time of forming the active complex ES^* , whereas if a specific Poisson process is significantly slower than the other processes, i.e., being a rate-limiting step, then the overall first-passage time distribution is Poisson in nature. Our experimental data shows a Gaussian-like distribution, suggesting that the ES^* formation time is not Poisson; therefore, it would not be possible that there is a significantly slow rate-limiting process that dominates the conformation diffusion motions. Mechanical motions rather than consecutive conformational motions have also been used to characterize the nonequilibrium conformation motions of proteins.^{51,52} Mechanical motion gives Gaussian-like distributions of first-passage time, and multiple consecutive conformational motions can equivalently give a Gaussian-like first-passage time distribution originated from a multiple Poisson step rate process convolution. Overall, our assumption of the rate processes having multiple steps and with a constant time constant is a reasonable and simple model to analyze our experimental data.

Conclusion

A bunching effect in nonequilibrium conformational motions associated with forming an active substrate–enzyme complex state has been revealed in single-molecule T4 lysozyme conformational dynamics. The consecutive multiple-step conformational fluctuation dynamics under the induction of the substrate–enzyme interactions gives rise to a bunching effect, a novel dynamic characteristic for the overall rate process of the enzymatic active complex formation. It is most likely that the bunching effect is a general property for the conformational dynamics of enzymes that are regulated and rate-limited by conformational motions in forming enzyme–substrate complex states. Typically, the functional conformation selection mechanism applies when bunching effects in the conformational dynamics exist. As strong experimental evidence of ordered

molecular conformational motions comes from conformational fluctuations under enzymatic reaction conditions, the bunching effect appearing from enzyme conformational motions has significant and general biological relevance and consequences associated with some profoundly important ramifications, including biological oscillations and self-organizations, temporal and spatial functionality and complexity, and biological rhythms.

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